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Biodegradation of Diesel Oil Using *Acinetobacter NCIMB 110507* and *Pseudomonas putida NAS 1(4)*

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Abstract
Characterization and screening of stock cultures for application in diesel degradation was undertaken. Two isolates from the stock cultures from University of Wolverhampton Culture Collection (UWCC) were selected based on their positive growth on streak plates containing hydrocarbon media and were isolated into pure culture. The isolates were characterized and their identities were reconfirmed by carrying out a different biochemical test. All the tests and the biochemical characteristics agreed with the expected results of each organism. The isolates consisted of two Gram-negative rods *P. putida NAS 1(4)*, and *Acinetobacter (NCIMB 110507*). The results showed that *Acinetobacter* and *P. putida* grew better in 0.5% and 1.0% concentration of the compounds. Optimum growth was observed at the temperature of 20^{0}C to 37^{0}C and no growth was observed at 40^{0}C. Also, as the concentration increased from 0.5% to 2.0%, the rate of growth on these compounds reduces. *Pseudomonas putida NAS 1(4)* had a better growth at 2.0 %v/v concentration of hexadecane, sodium benzoate, and diesel oil as compared to *Acinetobacter*. The minimum inhibitory concentration (MIC) for *P. putida* was more than 50% for all the compounds and the MIC for *Acinetobacter NCIMB 110507* was 12.50 % for Sodium Benzoate and more than 50% for hexadecane and diesel oil. *Acinetobacter* had better growth at 20^{0}C having the highest optical density of 1.77 and percentage TPH degradation of 85.2% after 144hrs of incubation, degradation was higher for *Acinetobacter* than for *P. putida NAS 1(4). Acinetobacter NCIMB 110507* degraded 100% most of the light fraction of diesel alkane at 20^{0}C (C8, C9, C10, C11, C14, C20, C22) and 64.31% for C19 while at 40^{0}C, the range of degradation was 1.18 to 100% as demonstrated by GC-FID chromatogram analysis. For *P. putida NAS 1(4)*, the range of degradation for C8-C22 at 20^{0}C was 0 to 35.0% while at 40^{0}C it was 0 to 21.0%.

Keywords: Biodegradation, Diesel Oil, Hydrocarbon, *Acinetobacter*, *Pseudomonas Putida*

Introduction

The components of petroleum hydrocarbons include volatile and non-volatile aromatic compounds and aliphatic fractions. Diesel oil is a complex petroleum hydrocarbon that is obtained during crude oil distillation, and is made up of low molecular weight alkanes and polycyclic aromatic hydrocarbons (Kaur, *et al*., 2015). The hydrocarbon composition of diesel fuel makes it toxic to the environment and its widespread application in human activity makes diesel fuel one of the most hazardous hydrocarbon pollutants (Muratova, *et al*., 2012). Koma *et al*. (2003), reported the percentage hydrocarbon fraction of diesel oil which includes saturated fraction (90.9%), cyclic paraffin (75.4%), normal paraffin (15.5%), and an aromatic fraction (9.1%). Owing to the significant environmental hazard posed by aromatic components, most of the recent studies have focused mainly on these components (Varjani, *et al*., 2017). However, addressing the removal of large quantities of aliphatic hydrocarbons is important to achieve remediation goals. Furthermore, the mid-length aliphatic hydrocarbons of diesel oil are less biodegradable due to non-polar, non-volatile, water-insoluble, and limited bioavailability properties (Abbasian, *et al*., 2015; Stroud, *et al*., 2007). Recent studies have shown that the mid-length C18-C22 hydrocarbons of diesel are not readily degraded as compared to other aliphatic hydrocarbons (Bajagain, *et al*., 2018).

Pollution with petroleum hydrocarbons is a major environmental problem. The environment may become contaminated with these hydrocarbons through various routes, such as leakage from underground storage tanks and pipelines, accidental spills during transportation, drilling sites and improper waste disposal practices (Hawelke, *et al*., 2016). The Spillage of diesel oil contaminates the natural environment such as air, water, and soil with hydrocarbons (Liu *et al*., 2016). This is a global concern that needs to be addressed, because they are toxic to all forms of life, mutagenic and carcinogenic (Cerniglia, *et al*., 2001). One Litre of used diesel oil is sufficient to pollute one million gallons of freshwater (United State Environmental protection Agency [USEPA], 1996). It also renders the environment unpleasant and creates a possible hazard to humans, animals, and vegetation (Adelowo, *et al*., 2006).

The contaminations of soils by hydrocarbons disturb biological and particularly microbiological, chemical, and physical properties of soils, including their wettability (Klamerus-Iwan, *et al*., 2015; Rodríguez-Rodríguez, *et al*., 2016). The hydrophobic nature of hydrocarbons can modify the wettability of the surface of soil particles and they thus contribute to soil water repellence when coating soil particles (Roy, *et al*., 1999). Soil water repellency affects hydrological and ecological soil functions by decreasing water infiltration, increasing surface runoff and erosion, and impeding plant growth (Doerr, *et al*., 2000). Water repellency of soils limits their water sorptivity and results in uneven moisture distribution, forming preferential water flow in the soil profile (Hewelke *et al*., 2016; Szatyłowicz, *et al*., 2007). The phenomenon of soil hydrophobicity has long been known and intensively studied in naturally occurring soils (Doerr, *et al*., 2000). Oil contamination strongly increases the hydrophobicity of the soil; it loses its ability to absorb and retain water, displacing the air from the soil pores and ultimately destroying the water and air regime, leading to enhanced surface runoff, erosion, and reduced soil moisture (Marín-García, *et al*., 2016).

Bioremediation is a new technique for the elimination and degradation of several environmental contaminants including engine oil (Medina–Bellver, *et al*., 2005). This can be accomplished by using capable microbes. Bioremediation is defined as the use of the biological potentials of microorganism and their processes to eliminate or reduce the concentration of environmental pollutants on material to levels that are regarded safe by the regulatory bodies of that country (Liu, *et al*., 2016; Medjor, *et al*., 2015).

Until now, a range of approaches has been established to treat diesel oil pollution. While many of the conventional physical and chemical methods are efficient, they are also costly and can cause recontamination via secondary contaminants (Hong, *et al*., 2005). Remediation of oil spill-affected areas by the use of microorganisms is cheaper for restoring the ecosystem for ensuring clean groundwater supplies. Several studies have described microorganisms with better oil-degrading capabilities isolated from normal habitats that were previously contaminated with oil (Hess, *et al.*, 1997).

Bioremediation employs microorganisms to remove hydrocarbon contaminants (Tyagi, *et al*., 2011). The indigenous microbial population plays an important role in biodegradation, but their performance usually declines when the concentration of pollutants is high (Imron, *et al*., 2020; Serrano, *et al*., 2008). Therefore, engineered remediation ways such as biostimulation (use of nutrients) (Xu, *et al*., 2022), bioaugmentation (use of exogenous microbes) (Guarino, *et al*., 2017), and intrinsic bioremediation (use of indigenous microorganisms) (Oyewole, *et al*., 2022) are sometimes used to meet the remediation time or goal. The species of *Acinetobacter* spp K-6 (Chaudhary, *et al*., 2020), *Arthrobacter* spp from Antarctica (Abdulrasheed, *et al*., 2020), *Pseudomonas* spp (Baig, *et al*., 2021), *Bacillus* spp (Rache-Arce, *et al*., 2022) and *Rhodococcus spp* (Varjani, 2017), has been widely applied for the degradation of hydrocarbons. There is existing research interest in the effectiveness of bioaugmentation and its capacity to enhance petroleum hydrocarbons biodegradation (Jiang, *et al*., 2016; Tao, *et al*., 2019). Therefore, the isolation and selection of bacterial strains with strong degradation ability and survival in a wide range of environmental conditions can be a key step in implementing the bioremediation of petroleum hydrocarbons.

The study aimed to determine the biodegradation efficiency of diesel oil using *Acinetobacter* NCIMB

110507 and *Pseudomonas putida* NAS 1(4) at different temperatures and concentrations. The biodegradation of diesel oil was monitored using gravimetric and gas chromatographic analysis coupled with a flame ionization detector to ascertain how the carbon fractions change after the biodegradation.

Materials and Methods

Chromatographic grade standard n- alkane (C_8 – C_{28}), tryptic soy agar (TSA), tryptic soy broth (TSB), ringers tablet, Kovac's reagent, Bushnell and Haas mineral salts reagents, H_2O_2. All chemicals/reagents were procured from Sigma (Aldrich, UK) unless otherwise indicated. The diesel fuel utilized in this researched was acquired from a local filling station in Wolverhampton, United Kingdom, and was stored in a tightly closed plastic container and sterilized by filtering through a Millipore 0.22µM pore size membrane filter.

Culture Media Preparation

Preparation of Bushnell and Haas Medium

The Bushnell and Haas mineral salt medium was prepared by using the recipe of Bushnell and Haas (1940). To prepare one thousand milliliters (1000 mL) of Bushnell and Haas medium (BHM), the following chemicals (KH_2PO_4, 1.0g; K_2HPO_4, 1.0g; NH_4NO_3, 1.0g; $MgSO_4.7H_2O$, 0.2g; $FeCl_3$, 0.05g; $CaCl_2.2H_2O$, 0.02g; and Agar 20g) were weighed separately into a 250 mL beaker and 100 mL of distilled water was added, the solution was mixed by a magnetic stirrer to ensure dissolution. The same method was used for the chemicals. After homogenization, the mixture was transferred into a 1000 mL volumetric flask using a thistle funnel, and more distilled water was added to make 1000 mL. The pH was adjusted to 7.0 ± 0.2 and was distributed into the required volume in a 250 mL conical flask, stoppered and wrapped with aluminium foil, then autoclaved at 121^0C for 15 minutes at 15lbsq/inch pressure.

Bushnell and Haas agar (BHA) medium was prepared by weighing 4g of agar into a 300 mL medical flask, 200 mL of BHM was added into the medical flask and was swirled to mix. The media was autoclaved at 121^0C for 15 minutes and was kept at 50^0C incubator until required.

Preparation of (TSA) and (TSB)

Tryptic soy agar was prepared (TSA) was prepared by weighing 40g/L or 8g of the TSA powder into a medical flask, 200 mL of distilled water was added to it and was mixed properly and autoclaved at 121^0C for 15 minutes. After sterilization, it was kept at a 50^0C incubator to cool to 50^0C after which a 20 mL aliquot of the molten media was poured aseptically into sterile Petri dishes and allowed to solidify. It was then preserved in the refrigerator until it is needed.

To make 200 mL TSB broth, 6 g of TSB powder was weighed into a 300 mL medical flask, 200ml of distilled was added to it and mixed properly before it was autoclaved. After sterilization, it was preserved at room temperature until needed.

Preparation Hexadecane Agar

To prepare 0.5%, 1%, and 2% hexadecane, Mixed BHM (20 mL) was poured into a 50 mL Erlenmeyer flask covered and wrapped around with an aluminium foil paper while the remaining mixture containing agar (180 mL) was autoclaved in the medical bottle. All were autoclaved at 121ºC for 15 minutes, 1.0 mL of membrane filtered (0.45µm) hexadecane was aseptically poured into the beaker containing 20 mL sterile BHM.

The beaker was then held in place in the chamber of a sonicator (Bandelin Sonoplus, Model 2200) by a holding cable and lever on the clamp stand. The flat-tip (VS70T) of the sonicator probe was carefully scrubbed using ethanol before being introduced to a depth of about 1 cm of the mixture. The sonicator was then continuously run for 2 minutes. This was repeated twice to create an emulsion. The homogenized mixture of sterile BHM with hexadecane were then aseptically poured into the autoclaved BH agar in the medical bottle and thoroughly mixed.

The molten mixture was then aseptically poured into sterile Petri dishes and left to harden. This procedure was repeated for 1% and 2% hexadecane. To prepare TSA containing 0.5%, 1.0%, and 2.0% hexadecane, the same method used for the preparation of hexadecane was adopted except that 20 mL BHM and 180 mL BHA was substituted with 20 mL TSB broth and 180 mL TSA.

Preparation of Diesel Fuel Agar

The same procedures described in the preparation of hexadecane was used for the preparation of BHA containing 0.5%, 1.0%, and 2.0% diesel fuel, to Prepare TSA containing 0.5%, 1%, and 2% diesel fuel. The same method used in the preparation of hexadecane was repeated except that 20 mL BHM and 180 mL BHA was replaced with 20 mL TSB broth and 180 mL TSA.

Isolation and Characterization of the Bacterial Cultures

Preparation of Bacterial Culture from University of Wolverhampton Culture Collection (UWCC)

The freeze-dried ampoules of bacteria isolates from UWCC were cultured on tryptic soy agar (TSA) plate. These were incubated at 37^0C for 24 - 48hrs, isolated bacteria colonies were subsequently transferred into fresh plates of TSA, and stored in the refrigerator at 4^0C for further procedure.

Characterization of Bacterial Isolates

The following tests described were used to confirm the identity of the screened and selected bacteria for hydrocarbon degradation.

Motility Test

A hanging drop preparation described by Tiwari *et al*. (2009) was used to determine the bacteria motility. One drop of bacteria culture is placed onto a clean scratch-free glass slide. Then a clean coverslip was carefully placed over the drop avoiding bubbles formation. The slide was examined first under x10 magnification followed by x40 and x100 magnifications and motility determined by observation.

Oxidase Test

The cells of bacteria were smeared across the surface of the Kovac's reagent impregnated filter paper with the aid of a glass rod and instant development of a dark purple colour showed a positive reaction (Standards Unit, Evaluations, and Standards Laboratory [SUESL], UK. 2007).

Catalase Test

One drop of 3% H_2O_2 (Sigma) was placed on a slide then a loop full of bacteria was introduced, an immediate effervescence indicated a positive result.

Screening Bacterial Isolates for Hydrocarbon Degradability

The bacteria isolated from UWCC were screened for hydrocarbon (diesel fuel, hexadecane, and sodium benzoate) degradation at temperatures range of 25^0C-40^0C of hydrocarbon concentrations of 0.5%, 1.0%, and 2.0%. These were carried out via culturing of the bacterial isolates on BHM and TSA plates containing hydrocarbon. The plates were incubated in the incubators at an average temperature of 25 ± 1^0C, 30 ± 1^0C, 37 ± 1^0C, and 40 ± 1^0C for a period up to 7 days.

Minimum Inhibitory Concentration (MIC) Test

The minimum inhibitory concentration of diesel fuel was determined for both the isolates using the method described by Wiegand *et al*. (2008). Doubling dilution (½ to 1/1056) of the diesel was diluted in a 96 microtiter plate using 150 µL of TSB and 150 µL of the diesel. Twenty microliters (20 µL) suspension of the test organism was added aseptically to the test wells and the positive control wells, no cells were added to the negative control wells and it was incubated at 37^0C for 24hrs. The optical density was measured using GEN 5 EL800 microtiter plate reader at 593nm.

Biodegradation of Diesel in Liquid Culture

Preparation of Standard Inoculum

The individual bacterium was cultured in BHM supplemented with 1% diesel for 18 h at 30°C in an orbit shaker at 150 rpm. Bacteria cells was harvested by centrifugation, rinsed three times in sterile ringers solution before being re-suspended in 5 mL sterile liquid Bushnell and Haas medium (BM) to yield an absorbance reading of 0.5 at 600 nm.

Biodegradation of Diesel in Shake Flask

The biodegradation of diesel in shake flasks was employed according to the method of Das and Mukherjee (2007). 100 mL of sterile BHM supplemented with 1% sterile diesel fuel in 250 mL Erlenmeyer flask was inoculated. 1 mL of the standard inoculum of each organism was inoculated and incubated at 20^0C and 40^0C for 7 days. Biodegradation was monitored by measuring the optical density at 600nm.

Extraction and Analysis of Diesel fuel by Gravimetric and GC

After the incubation period described in the shake flask, the supernatant phase was separated using a separating funnel after centrifuging the culture broth at 5,000 rpm for 20 minutes. The remaining diesel fuel was extracted by adding 20 mL of hexane and shaking thoroughly according to the method described by Mendri and Lin (2007). The extracted

phase was then collected in a beaker of known weight and the weight difference was noted after evaporating the solvent. The amount of residual oil was calculated from the weight difference. The percentage of oil degraded was calculated as follows (Bhattacharya & Biswas., 2014).

% of oil degraded = [(Wt. of test oil sample) – (Wt. of residual oil sample)]/ (Wt. of test oil sample) × 100

Gas Chromatographic Analysis

Biodegradation of diesel fuel was monitored by quantitative gas chromatography according to the method described by Michaud, *et al*. (2004). Hexane extracts of the remaining diesel oil sample (1 µL) was injected for analysis into TRACE 1300 series gas chromatographs Thermo Scientific equipped with a SE 54 capillary column (15m x 0.32mm i.d, 0.45µ film thickness) and flame ionization detector (FID). Helium gas was used as a carrier gas (1 bar). The oven was programmed as follows: 50^0C (5 minutes) then increase to 280^0C at the rate of 10^0C /minutes, then isothermal for 10 minutes; injector and detector temperature was both maintained at 280^0C.

Results and Discussion

Characterization of Stock Culture from University of Wolverhampton culture collection (UWCC)

Characterization and screening of stock cultures for application in diesel degradation was undertaken. Two isolates from the stock cultures from UWCC were selected based on their positive growth on streak plates containing hydrocarbon media and were isolated into pure culture. The isolates were characterized and their identities were reconfirmed by carrying out the different biochemical tests according to Bergey's manual of determinative bacteriology, 9th edition (Holt & Krieg., 1994). All the tests and the biochemical characteristics agreed with the expected results of each organism. The isolates consisted of two Gram-negative rods (*Ps. putida*, and *Acinetobacter*) all were catalase-positive, motile, and oxidase-positive except *Acinetobacter* which is oxidase negative as depicted in Table 1.

Table1: Morphological, Biochemical and characterization of Acinetobacter and *Pseudomonas Putida* (+) positive, (-) negative

Characteristics	*Acinetobacter* NCIMB 110507	*Pseudomonas putida* NAS 1(4)
Gram staining reaction	-	-
Morphology	Rods	Rods
Growth temperature range (ºC)	10-42	10-45
Motility	+	+
Catalase/Oxidase	+/-	+/-

Screening Bacteria for Application in Diesel Degradation

The results for the screening of the two organisms for hydrocarbon degradability on solid media at different concentrations of hydrocarbon (0.5, 1.0, and 2.0) percent diesel oil, hexadecane, and sodium benzoate are presented in Table 2: A, B, and C respectively.

The results showed that *Acinetobacter* and *P. putida* grew better in 0.5% and 1.0% concentration of the compounds, optimum growth were observed at the temperature of 25^0C to 37^0C and no growth was observed at 40^0C, Table 2: (A and B). As the concentration increases, (Table 2. C), the growth of the organisms reduces, this might be due to the inhibitory effects of the compounds at high concentrations. The growth of these isolates on different concentrations of the hydrocarbon showed that concentrations affected the growth. Thus, as the concentration increased from 0.5% to 2.0% the rate of growth on these compounds reduces (Table 2). Koolivand *et al*. (2022) reported that petroleum degrading bacteria grew well at 1%-3% level of crude oil in BHM. However, higher concentration of (4 to 5%) resulted in reduced growth of the organisms since high amounts of the petroleum hydrocarbons may be toxic to the organisms. Several studies show that the, extend and direction of the effects of multiple substrates like diesel oil on biodegradation kinetics depend on the chemical concentration (Dasai, *et al*., 2008; Knightes, & Peters., 2006; Stringfellow, & Aitken., 1995). At

high concentrations, most chemical can be toxic and inhibit metabolic processes.

Table 2: Screening of *Acinetobacter* NCIMB 110507 and *Pseudomonas putida* NAS 1(4) for hydrocarbon degradation at varying temperatures and concentrations of the compounds (A) 0.5%, (B) 1 %, an (C) 2%

(Concentration)	25^0C				30^0C				37^0C				40^0C			
Bacteria	TSA	HD	SB	DO	TSA	HD	SB	DO	TSA	HD	SB	DO	TSA	HD	SB	DO
A (0.5%)																
Acinetobacter	++	+	+	+	+++	++	++	+	++	+	+	+	+	-	-	-
P. putida	++	+	+	+	+++	++	++	+	++	+	+	+	+	-	-	-
B (1.0%)																
Acinetobacter	++	+	+	+	+++	++	++	++	++	+	+	+	+	-	-	-
P. putida	++	+	+	+	+++	++	++	++	++	+	+	+	+	-	-	-
C (2.0%)																
Acinetobacter	++	+	+	+/-	+++	+	+	+	++	+	+	+	+	-	-	-
P. putida	++	+	+	+	+++	++	++	++	++	+	+	+	++	-	-	-

Note: (+++) growth in clusters, (++) heavy growth, (+) positive growth, (+/-) light growth and (-) no growth, TSA (Tryptone soya agar), HD (hexadecane) SB (sodium benzoate) DO (Diesel oil), Media was incubated for a period of 48 h (for the TSA) and 144 h (for HD, DO, SB)

Shukor *et al*. (2009) reported that degradation is generally poor at concentrations higher than 1.5%. v/v. Only *Pseudomonas putida* had a better growth at 2.0 %v/v concentration of hexadecane, sodium benzoate, and diesel oil, this might be due to the presence of hydrocarbon degradation genes such as alkane degradation genes (*Gpol* and *AlkB*), aromatic hydrocarbon degradation gene (*XylE*), and polyaromatic hydrocarbon degradation gene (*ndoB*), (Margesin, *et al*., 2003). Weak growth was observed for both organisms at a high concentration of 2.0% and high temperature of 40^0C (Table 2. C), possibly due to poor enzymes activities (Merino & Bucala. 2007), slow metabolism (Dasai, *et al*., 2008; Koolivand, *et al*., 2022; Knightes, & Peters., 2006; Stringfellow & Aitken. 1995), or different pathways (Das & Chandra, 2011). The growth of these organisms was evaluated at a range of temperatures from 20^0C to 40^0C (Figure 1), and it was found that the temperature effected the growth and metabolism of bacteria. The optimum temperature for all the selected bacteria was 30^0C. The temperature optima for bioremediation according to Si-Zhong *et al*. (2009) are usually 15-30^0C for aerobic process and 25-35^0C for the anaerobic process.

The rate of biochemical reaction in cells increases with an increase in temperature up to a maximum above which the enzyme's activities cease to function due to protein denaturation Trasar-Cepeda, *et al., (*2007), Abid *et al.* (2007) and Kuntz *et al*. (2008) reported that temperature affects bacterial metabolism, growth rate, and the physicochemical characteristics of the hydrocarbon, most organisms grow best at mesophilic temperature of 20^0C to 40^0C (Figure1).

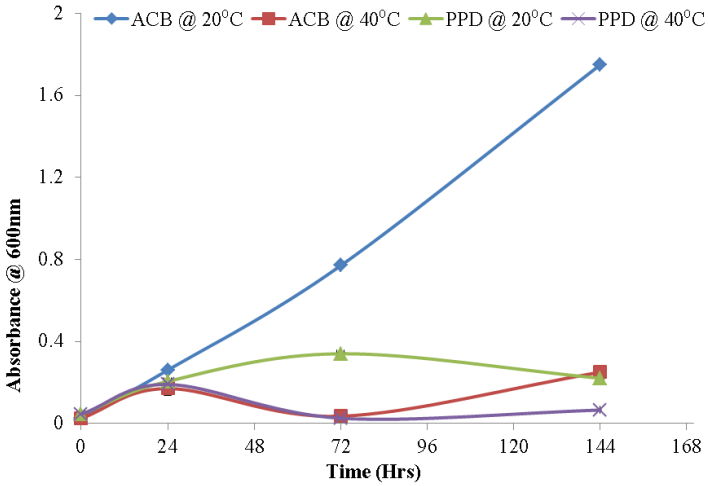

Figure 1: Growth absorbance at 600nm for Acinetobacter and *P. putida* with time at 20^{0}C and 40^{0}C in shake flask containing 1% diesel in BHM

Another effect of temperature was demonstrated by Munna *et al.* (2015) who reported that the cells of *Ps. putida* grow vigorously at 30^{0}C while growth was found to decline at a lower temperature of 27^{0}C and above 33^{0}C which might be due to enzymes inactivation. However, the changes in morphology were inconsequential, but at 40^{0}C, the cells were completely lost their ability to grow after 48hrs. The result in Figure 1 indicated that *Acinetobacter* and *P. putida* grew better at 20^{0}C as compared to their growth at 40^{0}C. This might be due to their optimum growth temperature which is between 20^{0}C to 30^{0}C and it might also be the reason why they grew poorly at 40^{0}C.

The MIC for different hydrocarbon compounds was determined in Figure 2. The MIC for *P. putida* was more than 50% for all the compounds in Figure 2A and the MIC for *Acinetobacter* was 12.50 % for Sodium Benzoate and more than 50% for hexadecane and
diesel oil in Figure 2B.

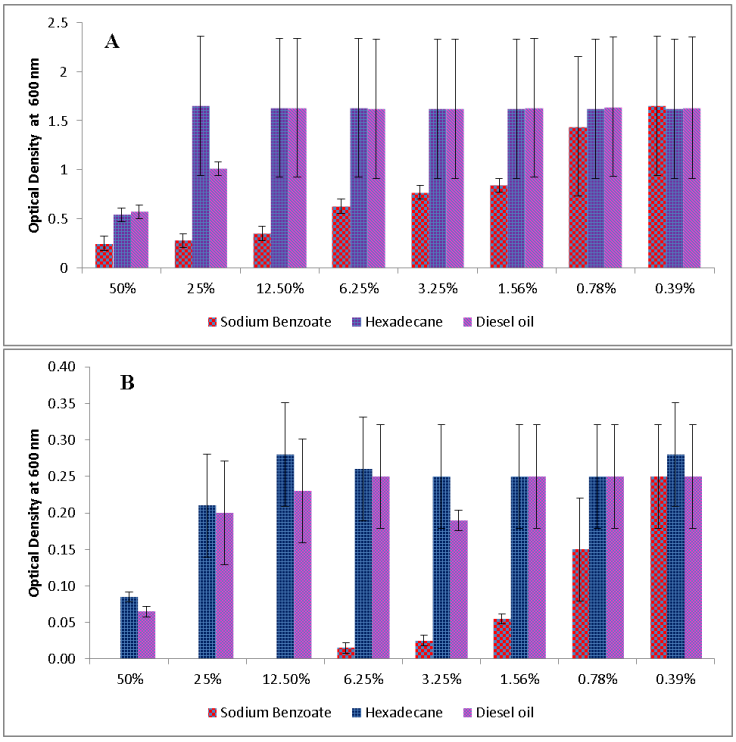

Figure 2: Minimum Inhibitory Concentration (MIC) of Sodium Benzoate, Hexadecane and Diesel Oil for (A) *Pseudomonas putida* and (B) *Acinetobacter* at Different Concentration (v/v) Growing in TSB at 37^{0}C for 24HRS.

The toxicity of diesel depends on its composition. *N*-alkanes of intermediate chain length of (C10-C24) are degraded most rapidly, short chain alkanes (<C9) are toxic to many microorganisms, because their water solubility results in increased uptake of the alkanes. High chain length alkanes are generally resistant to biodegradation. Branching in alkanes generally reduces the rate of biodegradation (Berthe-Corti & Fetzner., 2002). Biologically active substance in terms of toxicity and bioaccumulation have Log Octanol/Water partition Coefficient (KOW) values from 2 to 6 (Trapp, *et al.,* 2001). Most hydrocarbon compounds fall within this range. Log KOW for n-hexane, n-decane and diesel are 2.91, 5.58 and > 3.3 respectively. Compounds with LOG POW value between these ranges are very toxic for microorganisms (De Smet *et al*. 1978; Osborne *et al.*, 1990), those with lower LOG POW values are considered more toxic than those with higher LOG POW values (Inoue & Horikoshi., 1989). Compounds with LOG POW below 1 is too low to allow compounds to cross the membrane and above 6 is very poorly water-soluble and, as it is not bioavailable, it cannot cause any toxic effect (Heipieper, *et al.,* 1996).

The growth of the isolates in a shake flask supplemented with 1% diesel oil as sole carbon and energy sources at 20^0C and 40^0C was carried out for seven as shown in days Figure 3. *Acinetobacter* had better growth at 20^0C having the highest optical density and percentage TPH degradation after 144hrs of incubation, this trend was also observed at 40^0C. Furthermore, this pattern of growth and extent of degradation was similar at 40^0C but at a lower rate, possibly due to the lag phase being prolonged (Scharzenbach, *et al*., 2005) and the activities of the enzymes being slowed down. Ludzack and Kinkead (1956) reported that temperature affects the rate of hydrocarbon degradation as consequences of enzymes inhibition, membrane toxicity and slow down of general metabolic activity. *Acinetobacter* was able to degrade 85.2% TPH after seven days' incubation at 20^0C (Figure 3B).

Nkem *et al.* (2016) also reported that *Acinetobacter* was able to degrade 58.1% TPH of diesel in 10 days at 32^0C. These differences may be attributed to the effects of temperature differences or enzymes activities or the dissolved oxygen and nutrients were depleted which may affect the rate of degradation. In this present study, the growth of *Acinetobacter* continued to grow exponentially up to 144hrs; it could be possible that the rate of growth and degradation would have increased with time. *P. putida* had better growth and degradation at 20^0C, the growth increased exponentially throughout the incubation period while at 40^0C the growth reached an exponential stage after 24hrs and start to decline. This could be explained by the fact that optimum temperature for growth of *P. putida* is between 20–30^0C as reported by Munna *et al.* (2015) this could affect the activities of the enzymes, rate of growth as well the rate of degradation will also be affected outside this range. In this study, it was shown that *P. putida* was able to degrade 58.0% TPH within seven days of incubation at 20^0C (Figure 3B). A similar study by Vinothini *et al*. (2015) showed that *P. putida* was able to degrade 65% TPH diesel within seven days.

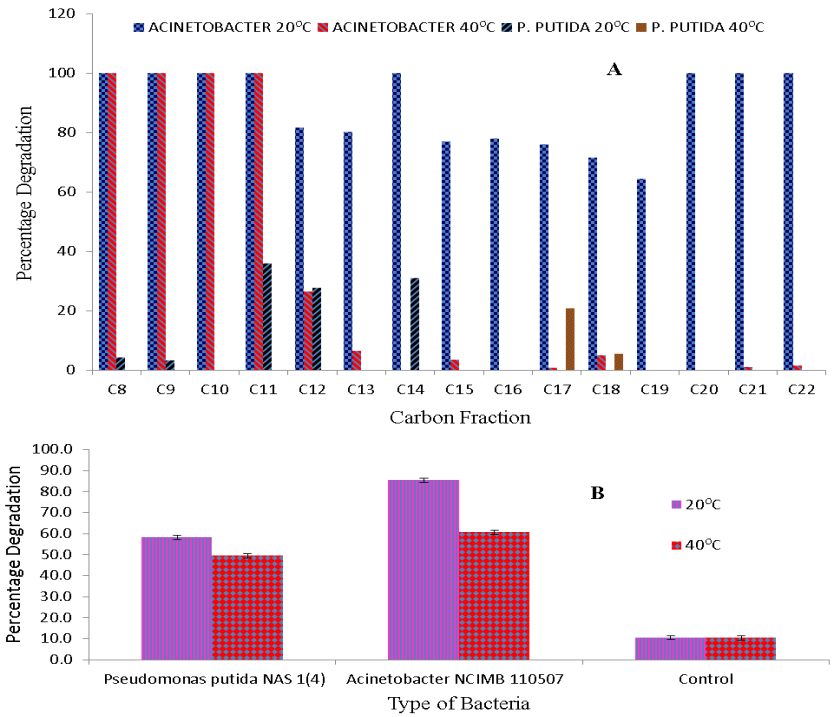

Figure 3: (A) individual n-alkane degradation and (B) Percentage total petroleum hydrocarbon (TPH) degradation of diesel oil in shake flask incubated at 20^{0}C and 40^{0}C for seven days by *Acinetobacter* NCIMB 110507 and *Pseudomonas putida* NAS 1(4).

Biodegradation of diesel was evaluated after 7 days of incubation at 20^{0}Cand 40^{0}C in shake flask. The result reveals that the degradation was higher for *Acinetobacter* than for *P. putida*. *Acinetobacter* degraded 100% most of the light fraction of diesel alkane at 20^{0}C (C8, C9, C10, C11, C14, C20, C22) and 64.31% for C19 while at 40^{0}C, the range of degradation was 1.18 to 100% as shown in Figure 3A and demonstrated by GC-FID chromatogram analysis in Figure 4D. For *P. putida*, the range of degradation for C8-C22 at 20^{0}C was 0 to 35.0% while at 40^{0}C it was 0 to 21.0% as shown in Figures 3A and 4C.

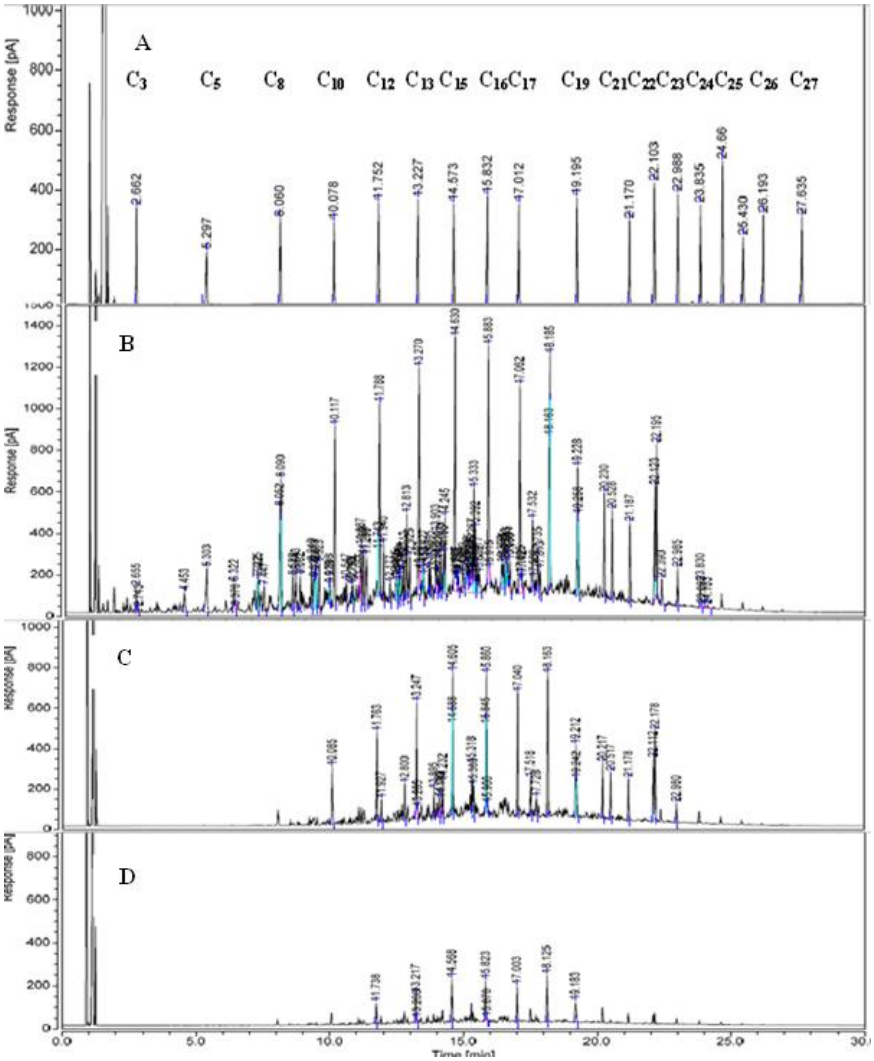

Figure 4: Chromatogram of (A) hydrocarbon standard; (B) diesel oil at T = 0; (C) degradation by *Pseudomonas putida* NAS 1(4); and (D) degradation by *Acinetobacter* NCIMB 110507.

Many researchers have reported that the extent to which diesel oil hydrocarbons are removed is connected closely to their molecular composition. Microorganisms preferentially degrade alkanes due to their simple structure compared to other forms of hydrocarbon (Verma, *et al*., 2006).

High solubility and bioavailability of short-chain alkanes to bacterial cell membranes which is toxic may be the reason for its incomplete degradation while incomplete degradation of long-chain alkane may be due to its membrane's low solubility, low bioavailability, and membrane toxicity to bacterial cells (Nkem *et al.* 2016). Variability in chain length and structure of hydrocarbon which requires different degradative enzymes and pathways explains why incomplete degradation is occurring (Das & Chandran. 2010).

The characteristics of mixed culture consisting of different types of bacteria with different enzymes derivatives will also affect the rate of biodegradation and the nutrient in the medium (Chemlal, *et al.* 2013). Excluding the loss due to abiotic depletion (C8 - C10) as indicated by the control, the rate of biodegradation of some lower diesel alkanes (C11-C13) was higher than the one of (C14-C21). This demonstrated that lower alkanes were first degraded and assimilated which led to an increase in degradation of higher alkanes. This may increase their growth rate and subsequent production of more hydrolyzing enzymes. Izquierdo *et al.* (2015) reported that the presence of lower alkanes in a mixture of hydrocarbon had a synergistic effect on the removal of polyaromatic hydrocarbon, increasing their rate of degradation by 73% and 59%

with and without alkane respectively. Degradation of branched or complex diesel oil fraction may produce toxic biodegradation intermediate that can inhibit the growth of diesel degrading organism which reduces further degradation (Frankenberger. 1992). As a result of this, more fractions from (C14-C21) had the lowest degradation, and C22 and C23 had the highest degradation rates for both organisms.

Conclusion

The biodegradation efficiency of diesel oil was higher at 20^0C than at 40^0C for both isolates and the decrease in hydrocarbon concentration reached more than 85% at 20^0C and 60% at 40^0C after 144 hours of incubation for *Acinetobacter*.

Recommendations

The results suggest the possible exploitation of these two strains in the future biotechnological processes. The organisms can be used directly for bioremediation in cold, tropical, and temperate diesel oil contaminated soil and marine environments.

References

Abbasian, F., Lockington, R., Mallavarapu, M., & Naidu, R. A. (2015). Comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. *Applied Biochemistry and Biotechnology.* 176, 670-699.

Abdulrasheed, M., Zakaria, N., Roslee, A. F. A., Shukor, M. Y., Zulkharnain, A., Napis, S., Convey, P., Alias, A. S., Rocha, G. G, & Ahmed, S. A. (2020). Biodegradation of diesel oil by cold-adapted bacterial strains of *arthrobacter* spp from Antarctica. *Journal of Antarctic science, 32*(5), 341-353.

Abid, A. A., Sarvajeet, S. G., Ritu, G., Guy, R. L., & Lee, N., (2014). *Phytoremediation: Management of Environmental Contaminants*, volume1. Springer, New York.

Adelowo, O. O., Alagbe, S. O., & Ayandele, A. A. (2006). Time-dependent stability of used engine oil degradation by cultures of Pseudomonas fragi and Achromobacter aerogens. *African Journal of Biotechnology*, *5*(24), 2476– 2479.

Bajagain, R., Park, Y., & Jeong, S. W. (2018). Feasibility of oxidation-biodegradation serial foam spraying for total petroleum hydrocarbon removal without soil disturbance. *Sci. Total Environment*, 626,1236-1242.

Baig, Z.T., Abbasi, S. A., Memon, A. G., Nas, M, & S00mro, A. F. (2021). Assessment of degradation potential of *pseudomonas* species in bioremediating soils contaminated with petroleum hydrocarbons. *Journal of Chemical Technology and Biotechnology*, *97*(2), 455-465. Doi.org/10.1002/jctb.6820

Berthe-Cort, & fetzner. (2002). Bacterial metabolism of n-alkanes and ammonia under oxic, suboxic, and anoxic conditions. *Acta Biotechnology, 22*, 299-336

Cerniglia, C.E., & Sutherland, J.B. (2001). *Bioremediation of polycyclic aromatic hydrocarbons by ligninolytic and non-ligninolytic fungi*. In Fungi in Bioremediation, G. M. Gadd, Ed., pp136–187, Cambridge University Press, Cambridge, U.K.

Chaudhary, D. K., Bajagain, R., Jeong, S. W, & Kim, J. (2020). Biodegradation of diesel oil and n-alkanes (C18, C20, and C22) by a novel strain Acinetobacter sp, K-6 in unsaturated soil. *Journal of environmental research, 25*(3), 290-298

Chemlal, R., Abdi, N., Lounici, H.,Drouich, N., Pauss, A., & Mameri, N. (2013). Modeling and qualitative study of diesel biodegradation using biopile process in sandy soil *International Biodeterioration. Biodegradation, 78*, 43-48

Das, N. & Chandran, P. (2011). Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. *Biotechnology Research International. pp* 1-13.

de Smet, M.J., Kingma, J & Witholt, B. (1978). The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of Escherichia coli. Biochim. Biophys. Acta 506, 64-80.

Deng, M.C Li, J., Liang, F.R., Yi, M., Xu, X.M., Yuan, J.P., Peng, J., Wu, C.F & Wang J.H.(2014). Isolation and characterization of a novel hydrocarbon-degrading bacterium Achromobacter sp. HZ01 from the crude oil-contaminated seawater at the

Daya Bay, southern China Marine. *Pollution Bulletin,* 83, 79–86

Desai, A, M., Autenrieth, R. L., Dimitriou-Christidis., P, & MacDonald, T. J. (2008). Biodegradation kinetics of select polycyclic aromatic hydrocarbon (PAH) mixtures by sphingomonas paucimobilis EPA505. *Journal of Biodegradation*, *19*(2), 223-233.

Doerr, S. H., Shakesby, R. A., & Walsh, R. (2000). Soil water repellency: its causes, characteristics and hydrogeomorphological significance. Earth-Science Reviews, *51*(1), 33–65.

Frankenberger, W.T. J. (1992). *The need for a laboratory feasibility study in bioremediation of petroleum hydrocarbons E.J. Calabrese, P.T. Kostecki (Eds.),* Hydrocarbon contaminated soils and groundwater, Lewis, Boca Raton, FL, 237–293

Guarino C, Spada V, & Sciarrillo R. (2017). Assessment of three approaches of bioremediation (natural attenuation, landfarming and bioaugmentation-assisted landfarming) for a petroleum hydrocarbons contaminated soil. *Chemosphere*, 170, 10-16.

Heipieper, H.J., Meulenbeld, G., van Oirschot, Q and de & Bont, J.A.M. (1996). Effect of environmental factors on the trans/cis ratio of unsaturated fatty acids in Pseudomonas putida S12. *Appllied. Environmental Microbiology*. *62*, 2773-2777

Hess, A., Zarda, B., Hahn, D., Haner, A., Stax, D., Hohener, P. & Zeyer, J. (1997). In situ analysis of denitrifying toluene and m-xylene degrading bacteria in a diesel fuel contaminated laboratory aquifer column. *Appllied. Environmental Microbiology, 63* (6), 2136–2141.

Hewelke, E., Szatyłowicz, J., Gnatowski, T., & Oleszczuk, R. (2016). Effects of soil water repellency on moisture patterns in a degraded sapric histosol. *Land Degradation and Development, 27*(4), 955–964.

Holt, J. G., Krig, N. R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (1994). Bergey's maual of determinative bacteriology (9th edn). Baltimore, Maryland:Williams and Wilkins.

Hong, J.H., Kim, J., Choi, O.K., Cho, K-S and Ryu, H. W. (2005). Characterization of a diesel degrading bacterium, *Pseudomonas aeruginosa* IU5, isolated from oil-contaminated soil in Korea. *World Journal of Microbiology and Biotechnology, 21*, 381–384

Imron, M. F, Kurniawan, S. B., Ismail, N. I., & Abdullah, S.R.S. (2020). Future challenges in diesel biodegradation by bacteria isolates: A review. *Journal of cleaner production*, 252, 119716

Inoue, A. & Horikoshi, K. (1989). A Pseudomonas thrives in high concentration of toluene. *Nature 338*, 264-266.

Izquierdo, A., Vila, J., Petit, C., Peyret, P., Koch, A. & Grifoll, M. (2015). Microbial populations and functions associated with the degradation of aliphatic and aromatic hydrocarbon oil fractions. *Journal of Biotechnology* [online]**,** 208pp. S51-S51.

Jian, Y., Paton, G. I., Semple, K. T., Pollard, S. J, & Coulon, F. (2016). Insights into the biodegradation of weathered hydrocarbon in contaminated soils by bioaugmentation. *Chemosphere, 161*, 300-307

Kaur, J., Adamchuk, V. I., Whalen, J. K., & Ismail, A. A. (2015). Development of an NDIR CO2 sensor-based system for assessing soil toxicity using substrate-induced respiration. *Sensors*, *15*(3), 4734–4748.

Klamerus-Iwan, A., Błońska, E., Lasota, J., Kalandyk, A., & Waligórski, P. (2015). Influence of oil contamination on physical and biological properties of forest soil after chainsaw use. *Water, Air, & Soil Pollution*, *226*(11), 389. https://doi.org/10.1007/s11270-015-2649-2.

Knightes, C. D, & Peters, C. A. (2006). Multisubstrates biodegradation kinetics for binary and complex mixtures of polycyclic aromatic hydrocarbons. *Journal of Environmental Toxicology and Chemistry*, *25*(7), 1746-1756

Koolivand, A., Abtahi, H., Parhamfar, M., Saeedi, R., Coulon, F., Kumar, V., Villasenor, J., Sartaj, M., Najarian, N., Shahsavani, M., Seyedmoradi, P., Rahimi, L, & Bagheri, F. (2022). The effect of hydrocarbon concentration on competition between oil-degrading bacteria and indigenous compost

microorganisms in petroleum sludge bioremediation. *Journal of environmental technology and innovation, 26*, ISSN. 2352-1864

Koma, D., Hasumi F.,Yamamoto E.,Ohta., Chung S.Y & Kubo, M. (2001). Biodegradation of long chain n-paraffin from waste oil of car engine by *Acinetobacter sp*. *Journal of Bioscience and Bioengineering*, *.91*, 94-96

Kuntz J, Nassr–Amellal N, Lollier M, Schmidt J. E, & Lebeau T. (2008). Effect of sludges on bacteria in agricultural soil. Analysis at laboratory and outdoor lysimeter scale. *Ecotoxicology and Environmental Safety, 69*, 277–288

Liu, Y., HU, X. & Liu, H. (2016). Industrial-Scale Culturing of the Crude Oil-Degrading Marine *Acinetobacter* Sp. Strain HC8-3S *International journal Biodeterioration and Biodegradation, 107* , 56-61

Ludzack, F.L & Kinkead D. (1956). Persistence of oily wastes in polluted water under aerobic conditions. *Ind. Engng Chem. 48*, 263-7

Margesin, R., Labbe, D., Schinner, F., Greer, C.W & Whyte L.G. (2003). Characterization of Hydrocarbon-Degrading Microbial Populations in Contaminated and Pristine Alpine Soils. *Applied And Environmental Microbiology, 69* (6), 3085-3092.

Marín-García, D. C., Adams, R. H., & Hernández-Barajas, R. (2016). Effect of crude petroleum on water repellency in a clayey alluvial soil. *International journal of Environmental Science and Technology, 13*(1), 55–64.

Medina-Bellver J. I., Mar´ın P., & Delgado A. (2005). Evidence for *in situ* crude oil biodegradation after the Prestige oil spill, *Environmental Microbiology*, *7*(6), 773–779.

Medjor, W. O., N. O. Namessan2, N. O., Wepuaka, C., Foshen, G. & Suleiman, A (2015). Bioremediation of surface water contaminated with hydrocarbons in a non-oil producing area *British Journal of Applied Science and Technology*, *8*(5), 484-493.

Merino, J, & Bucala, V. (2007). Effect of temperature on the release of hexadecane from soil by thermal treatment. *Journal of Hazard Material, 143*, 455-461

Michaud, L. & Bruni, V. (2004). The biodegradation efficiency on diesel oil by two psychrotrophic antartic marine bacteria during a two month-long experiment. *Marine pollution bulletin, 46*(5-6)

Munna, S. M. D., Tahera, J. & Noor, R. (2015). Survival of bacillus spp SUBB01 at high temperatures and a preliminary assessment of itsability to protect heat-stressed Escerichia coli cells. *BMC research notes, 8*, 637

Nkem, B.M., Halimoon, N., Yusoff, F.M., Johari, W.L.W., Zakaria, M.P., Medipally, S.R. & Kannan, N. (2016). Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of Cellulosimicrobium cellulans and Acinetobacter baumannii from tarball at Terengganu beach, Malaysia. *Marine pollution Bulletin, 107*(1), pp. 261-268

Osborne, S.J., Leaver, J., Turner, M.K & Dunnill, P. (1990). Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Journal of Enzyme Microbiology and Technology*, 12, 281-291.

Oyewole, C. S., Akinlade, O. I., Abdulwasiu, Y. O., Kelechi, A. O. & Okoro, M. E. (2022). Biodegradation of selected petroleum hydrocarbon using indigenous microorganisms. *Research square*, *1*(2).doi.org/10.21203/rs.3.rs-1267852/vi

Rachie-Arce, D. C., Machacado-Salas, M, & Rosero-Garcia, D. (2022). Hydrocarbon degrading bacteria in Colombia: *Systematic review*, *33*(22), 99-116

Rodríguez-Rodríguez, N., Rivera-Cruz, M. C., Trujillo-Narcía, A., Almaráz-Suárez, J. J., & Salgado-García, S. (2016). Spatial distribution of oil and biostimulation through the rhizosphere of Leersia hexandra in degraded soil. *Water, Air, and Soil Pollution, 227*(9), 319. https://doi.org/10.1007 /s11270-016-3030-9.

Roy, J. L., McGill, W. B., & Rawluk, M. D. (1999). Petroleum residues as water-repellent substances in weathered nonwettable oil-contaminated soils. *Canadian Journal of Soil Science, 79*(2), 367–380.

Schwarzenbach, R. P., Gschwend, P. M, & Imboden, D. M. (2005). *Biological transformations*. In Environmental Organic Chemistry, John Wiley & Sons, Inc:Hoboken, NJ, pp. 687-773

Serrano A, Gallego M, González, J. L, & Tejada M. (2008). Natural attenuation of diesel aliphatic hydrocarbons in contaminated agricultural soil. *Environmental Pollution,151*, 494-502.

Shukor, M., Hassan, N., Jusoh, A., Perumal, N., Shamaan, N., MacCormack, W, & Syed, M. (2009). Characterization of a diesel degrading strain isolated from a hydrocarbon contaminated site. *Journal of Environmental Biology 30*, 1-6.

Si-Zhong, Y., Hui-Jun, J., Zhi, W., Rui-Xia, H., Yan-Jun, J., Xiu-Mei, L & Shao-Peng, Y. (2009). Bioremediation of Oil Spills in Cold Environments: *A Review. Pedosphere, 3*, 371-381.

Stringfellow, W. T, & Aitken, M. D (1995). Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading *pseudomonads*. *Applied Environmental Microbiology*, *61*(1), pp. 357-362.

Stroud, J. L., Paton, G. I., & Semple, K. T. (2007). Microbe-aliphatic hydrocarbon interactions in soil: Implications for biodegradation and bioremediation. *Journal of Applied Microbiology*, *102*,1239-1253.

Szatyłowicz, J., Gnatowski, T., Szejba, D., Oleszczuk, R., Brandyk, T., & Kechavarzi, C. (2007). Moisture content variability in drained fen soil. In T. Okruszko, E. Maltby, J. Szatyłowicz, D. Swiatek, & W. Kotowski (Eds.), Wetlands monitoring, modelling and management (pp. 113–120). London: Taylor and Francis Group.

Tao, K., Zhang, X., Chen, X., Liu, X., Hu, X, & Yuan, X. (2019). Response of soil bacterial community to bioaugmentation with a plant residue-immobilized bacterial consortium for crude oil removal. *Chemosphere*, 222, 831-838

Trapp, S., Köhler, A., Larsen, L., Zambrano, K. & Karlson, U. (2001). Phytotoxicity of fresh and weathered diesel and gasoline to willow and poplar trees. *Journal of Soils Sediments, 1*, 71-76.

Trasar-Cepela C., Gil-Sotres F & Leiros M. C. (2007). thermodynamic parameters of enzymes in grassland soils from Galicia, NW Spain. *Journal of Soil Biology and Biochemistry, 39*, 311-319

Tyagi M, da Fonseca MMR, & de Carvalho C.C.R. (2011). Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation*, 22,231-241.

USEPA. (1996). Recycling used oil: What can you do? Cooperation Extension Services ENRI, *317*, 1 –2.

Varjani, S. J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresourse Technology*, 223, 277-286.

Verma, S., Bhargavaa, R., & Pruthi, V. (2006). Oily sludge degradation by bacteria from Ankleshwar, *India International Biodeterioration and Biodegradation., 57*, 207–213

Vinothini, C., Sudhakar, S., & Ravikumar, R. (2015). Biodegradation of petroleum and crude oil by Pseudomonas putida and Bacillus cereus. *International journal current microbiology and applied sciences, 4*(1), 318-329

Xu, J., Sun, Y., Tian, G., Li, X, & Yian, Z. (2022). Fast biodegradation of of long alkane by enhancing bacteria performance rate by peroxidation. Journal of environmental management, 301(1), 113933, ISSN. 0301-4797